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Review

Assembly and overexpression of membrane proteins in *Escherichia coli*

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Abstract

The bacterium *Escherichia coli* is one of the most popular model systems to study the assembly of membrane proteins of the so-called helix-bundle class. Here, based on this system, we review and discuss what is currently known about the assembly of these membrane proteins. In addition, we will briefly review and discuss how *E. coli* has been used as a vehicle for the overexpression of membrane proteins.

Keywords: *Escherichia coli*; Overexpression; Membrane protein

1. Introduction

Membrane proteins account for 20–25% of all open reading frames in sequenced genomes, and they fulfil a wide range of central functions in the cell, from ion conductance and nutrient uptake to intra- and intercellular signalling and cell–cell interactions [1]. The importance of membrane proteins is probably best illustrated by the fact that at least 50% of all drug targets are membrane proteins. Our knowledge of the assembly of this very important class of proteins is still poor, and functional and structural studies of membrane proteins have been strongly hampered due to difficulties in overexpressing them in high yields.

Here we will review what is currently known about the assembly of membrane proteins in the bacterium *Escherichia coli*, which is one of the most popular model systems to study the assembly of membrane proteins of the helix-bundle class. For the sake of clarity, in this review we will only deal with the assembly of membrane proteins of the helix-bundle class (i.e., inner membrane proteins, which we hereafter will refer to as membrane proteins) and not the β-barrel outer membrane proteins. Finally, we will briefly review and discuss how *E. coli* has been used as a vehicle for the overexpression of membrane proteins.

2. Assembly of membrane proteins in *E. coli*

2.1. Translocation of proteins across the inner membrane

Before we give an overview of what is known about the assembly of membrane proteins in *E. coli*, we will briefly discuss the translocation of secretory proteins across the inner membrane. This is necessary for a good understanding of the assembly of membrane proteins. Almost all components of the machinery involved in the translocation of secretory proteins across the inner membrane of *E. coli*, the so-called Sec machinery, were initially identified in genetic screens [2]. Biochemical studies have further characterized the Sec machinery [3–5]. Secretory proteins are kept in a translocation competent state (i.e., noncompletely folded state) by the chaperone SecB. The SecB–secretory protein complex is targeted at a late stage during translation or after translation to the inner membrane, where the secretory protein is delivered at the Sec translocase. The Sec translocase mediates the translocation of secretory proteins across the inner membrane. The core of the Sec translocase consists of the integral membrane proteins SecY, SecE, and SecG, and the peripheral subunit SecA, which exists as a dimer. SecYEG forms together with SecA a molecular machine that drives the stepwise translocation of secretory proteins across the inner membrane driven by the proton-motive force and ATP hydrolysis.

How the Sec components form a functional Sec translocase is matter of a vivid debate. Electron-microscopic

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examination of purified translocase indicated that under translocation conditions, four SecYEG heterotrimers assemble, catalyzed by dimeric SecA, into one large protein complex, forming a protein-conducting channel [6]. In contrast, a monomeric translocon was proposed based on co-immunoprecipitation and cross-linking experiments [7]. Based on combined 2D-crystallography, analytical ultracentrifugation, and native gel electrophoresis experiments, it was proposed that the active Sec translocase is comprised of a SecYEG dimer in the absence of SecA [8–10].

SecD, SecF, and YajC are additional nonessential translocase subunits that facilitate the translocation reaction. They form a subcomplex that under certain conditions can be co-purified with the SecYEG complex. SecDFYajC has been implicated in diverse processes like SecA functioning, release of translocated proteins, and also the maintenance of the proton-motive force across the inner membrane [3]. However, a recent study has questioned the role of SecDFYajC in maintaining the proton-motive force [11].

The assembly of membrane proteins in *E. coli* was initially studied in Sec mutant strains selected for protein secretion defects, and with the SecA inhibitor sodium azide. These studies did not point to a role of the Sec machinery in the assembly of membrane proteins, except for large domains of membrane proteins that have to cross the inner membrane [12,13]. However, it should be stressed that the strains that were used had been selected for secretion defects rather than membrane protein assembly defects. Furthermore, sodium azide only partially inhibits the activity of the Sec translocase subunit SecA.

In vivo studies using improved conditional Sec strains and in vitro studies using cross-linking and reconstitution techniques have pointed to a more prominent and general role for the Sec translocase in membrane protein assembly.

2.2. The SRP targeting pathway

In *E. coli*, most membrane proteins are targeted to the inner membrane via the S(ignal)R(ecognition)P(article) targeting pathway [14,15]. The SRP targeting pathway in *E. coli* is homologous to the SRP targeting pathway that targets both secretory and membrane proteins to the endoplasmic reticulum (ER) membrane in eukaryotes [16,17]. The eukaryotic SRP binds during biosynthesis to the N-terminal signal sequence of secretory and membrane proteins when it is just exposed outside the ribosome. Further translation is inhibited until the SRP contacts its receptor at the ER membrane, and subsequently dissociates from the nascent chain. The core of the eukaryotic SRP consists of a GTP-binding protein, the 54-kDa subunit (SRP54), and the 7SL RNA. They participate in a larger complex that binds to the signal sequence via SRP54. At the ER membrane, the SRP makes contact with its receptor subunit S(ignal recognition particle)R(eceptor)α, which is tethered to the membrane via the integral membrane subunit SRβ. Both SRα and SRβ are GTP-binding proteins.

In *E. coli*, Ffh (*F*ifty-*f*our *h*omolog), 4.5S RNA, and FtsY are homologous to the eukaryotic SRP54, 7SL RNA, and SRα, respectively. Depletion of Ffh, 4.5S RNA and FtsY does not affect the translocation of most secretory proteins across the inner membrane, which does not mean that the SRP pathway cannot target secretory proteins to the inner membrane [18,19]. The preferred substrates for SRP-mediated targeting appear to be membrane proteins [15,20]. Biogenesis of most (of only a limited test set of) membrane proteins is strongly hampered upon the depletion of Ffh, 4.5S RNA, and FtsY, which might explain the essential nature of these components. Recently, genetic screens were successfully employed where Ffh, 4.5S RNA, and FtsY were identified as factors that are involved in the targeting of membrane proteins to the inner membrane [21,22]. Finally, cross-linking studies have demonstrated that the *E. coli* SRP preferentially binds to particularly hydrophobic targeting signals present in the N-terminus of nascent membrane proteins [23,24].

In contrast to its eukaryotic counterpart, FtsY is located both in the cytoplasm and at the inner membrane [25] and can already interact with ribosome/nascent chain/SRP complexes in the cytosol. Consequently, FtsY might play a direct role in the targeting of these complexes. The mechanism and regulation of membrane association of FtsY is still enigmatic but appears complex, involving both protein and direct lipid contacts [14,26–28]. Upon interaction with membrane lipids, the GTPase activity of FtsY (and probably also of the bound SRP) is stimulated, which might result in the dissociation of the targeting complex. The released nascent chain is then transferred to the insertion site in a process that is poorly defined in both prokaryotic and eukaryotic cells.

In vitro studies using artificially truncated nascent chains suggested that the *E. coli* SRP can mediate co-translational targeting, but the in vivo relevance of these observations is still uncertain [29]. To enable co-translational targeting, the mammalian and yeast SRPs slow down translation upon interaction with the nascent chain. However, the SRP subunits responsible for this feature have no homologs in *E. coli.* Therefore, despite a recent claim that the *E. coli* SRP can arrest translation [30], it is still generally assumed that it cannot. A posttranslational role of the *E. coli* SRP cannot be excluded, and has a precedent in chloroplast SRP that interacts posttranslationally with thylakoid membrane proteins that are imported from the cytosol. Notably, the *E. coli* SRP is essential for the targeting of a model membrane protein with only one transmembrane segment (signal anchor sequence) that is located at the very C-terminus of the protein, suggesting a posttranslational mode of targeting [31,32].

2.3. Are there alternative membrane protein targeting pathways?

In *E. coli* cells completely depleted for SRP, assembly of SRP-dependent membrane proteins is not completely blocked [33,34]. How can these membrane proteins be

assembled in the absence of a functional SRP targeting pathway? Possible alternative mechanisms to explain this include mRNA-, ribosome-, and chaperone-mediated targeting.

It has been shown that the large subunit of the mammalian ribosome can remain associated with the ER membrane after termination of translation [35–37]. This large subunit can, if it is programmed with mRNA encoding secretory or membrane proteins, recruit a small subunit and subsequently mediate the insertion and translocation of natural SRP substrates in an SRP-independent fashion. This would imply mRNA targeting, which seems to be widespread in eukaryotes [38,39]. Interestingly, recent studies strongly suggest that mRNA targeting also occurs in bacteria [40,41].

A role for FtsY in the general targeting of ribosomes to the *E. coli* inner membrane has also been postulated, but the fate and function of these targeted ribosomes awaits further analysis [27,42]. In addition, the affinity of ribosomes for the translocase appears to be a conserved feature that also applies to *E. coli* [43]. This affinity, together with an exposed targeting signal, might be sufficient to target a subpopulation of membrane proteins to the translocase in the absence of SRP.

Finally, it has been suggested, based on an in vitro study, that in *E. coli*, the chaperone GroEL can mediate the posttranslational targeting of the membrane protein lactose permease [44]. Interestingly, evidence has been presented that GroEL can make a link with the inner membrane by interacting with the Sec translocase component SecA [45]. If GroEL indeed would play a role in membrane protein targeting, one would expect that upon SRP-depletion, the expression of GroEL would be upregulated. However, this is not the case [46].

2.4. Membrane insertion

As mentioned before, until recently, it was generally assumed that in *E. coli*, the Sec translocase was only involved in the translocation of large periplasmic domains of membrane proteins across the inner membrane. However, detailed biochemical analysis of the insertion of membrane proteins into the ER membrane in eukaryotes initiated a reevaluation of the role of the Sec translocase in the assembly of membrane proteins in *E. coli*.

At the ER membrane, the SRP is released from the arrested nascent polypeptide in a GTP-dependent process that requires both subunits of the SR [47] and the translocon, the Sec61 complex [48]. The nascent chain, freed of SRP, enters the translocon that is sealed to the ribosome to form one continuous channel to allow a tightly coupled translation/translocation reaction. A cryo-electron microscopy study has provided us with a snapshot of this process [49].

The core of the Sec61 complex consists of the Sec61α, Sec61β, and Sec61γ components. The Sec61α and Sec61γ components are homologous to the bacterial Sec translocase components SecY and SecE, respectively [16]. There is no homologue of the Sec translocase component SecA in the Sec61 translocon, which may be related to different mechanisms to energize insertion and translocation. On the other hand, the ER translocon contains an accessory component TRAM, which has no structural homologue in *E. coli*. Biochemical and electron microscopy studies indicate that purified Sec61 heterotrimers form oligomers and each oligomer contains three to four Sec61 heterotrimers that constitute the functional translocation channel, which is approximately 40–60 Å wide during translocation [50,51]. The aforementioned cryo-electron microscopy study points to a Sec translocon consisting of three Sec61 heterotrimers, but to a translocation channel that is considerably less wide than 40–60 Å [49]. This may reflect the dynamic nature of the translocon [52].

Oligomer formation is stimulated by the association of the Sec61 heterotrimer subunit Sec61α with ribosomes [48,50], which is mediated by the 28S rRNA of the large ribosomal subunit [43]. During the early stages of nascent chain insertion, the Sec61 channel is blocked at the lumenal side by Bi(nding)P(rotein), which maintains the permeability barrier at rest [53]. In addition, it has been suggested that BiP functions as a molecular ratchet, rather than a ‘pulling force’, which assists polypeptide chains to cross the membrane [54]. It has been suggested that transmembrane signalling may play a role in mediating a concerted action of factors that are involved in the membrane protein assembly process on either side of the membrane [55].

Hydrophilic polypeptides are translocated across the membrane. They can be glycosylated and partially folded at the lumenal side of the membrane while still attached to the ribosome at the cytosolic side. Hydrophobic transmembrane segments get trapped in the translocon, and at some stage move out into the lipid bilayer [56]. The exact mechanism by which this is achieved is not clear, but appears to differ depending on the nature, number, and orientation of the transmembrane segments (a discussion on this area of the assembly of membrane proteins can be found in Ref. [52]).

Genetic studies hinted first at an important role of the *E. coli* Sec translocase components SecA, SecE, and SecY in the insertion of membrane proteins [57–59]. In vitro cross-linking studies indicate that membrane proteins that are targeted by the SRP/FtsY pathway insert at the SecAYEG translocase that is also used for the translocation of substrates of the SecB targeting route. Nascent model membrane proteins of different topology and complexity were released from the SRP after docking of FtsY at the membrane, and inserted into the membrane close to SecY, and, depending on the model protein, to SecA [60–65]. The mechanism of transfer of the nascent chain from the SRP to the translocase is still enigmatic (like in eukaryotic systems) but could involve yet unidentified direct interactions of FtsY and the SRP with the translocase. Interestingly, the *Bacillus subtilis* Ffh appears to have affinity for SecA in an in vitro

binding study [66]. Alternatively, the affinity of ribosomes for the translocase might contribute to the transfer reaction [43]. It is also possible that the membrane surrounding of the translocase is enriched in acidic phospholipids that bind FtsY. In this respect, it is noteworthy that SecA, like FtsY, also preferentially binds to acidic phospholipids. In *E. coli*, in contrast to eukaryotes, the presence of the Sec translocase is not strictly required for the release of the nascent chain from the SRP [67]. Photo cross-linking studies using the model membrane protein FtsQ, which has one transmembrane segment and its N-terminus in the cytoplasm and its C-terminus in the periplasm, have shown that the earliest interactions of the transmembrane segment with the membrane involve both SecY and lipids [63]. These interactions are detected when the transmembrane segment is not even fully exposed outside the ribosome, reminiscent of the situation in the ER described above, suggesting a conserved mechanism of insertion. It remains to be demonstrated whether the insertion takes place in the interior of the translocation channel (to which the lipids would have access) or between the interface of the translocase and the lipid surroundings [52]. The hydrophilic regions of membrane targeted nascent FtsQ were shown to have extensive contact with both SecA and SecY. In contrast, mannitol permease (MtlA), which is a polytopic membrane protein, was found to contact only SecY in a similar approach, which might be related to the presence of a large periplasmic loop in FtsQ and not in MtlA, that does not require SecA for its assembly in vivo [62,68].

It has been suggested (see also above) that SecA triggers the oligomerization of SecYEG trimers into a functional translocation unit [6]. For SecA-independent membrane proteins like MtlA, bacterial ribosomes, rather than SecA, might be the trigger for the oligomerization of SecYEG trimers. Furthermore, the physical link between the ribosome and the translocase may serve as a handle to push the nascent chain into the translocon obviating the need for SecA to energize membrane protein insertion [43,49]. Notably, SecA is not required for the initial membrane insertion of FtsQ [67], whereas it is essential for the complete assembly of the protein in vivo [63]. It is unclear if SecA would not always be available at the translocation site, when and how the need for SecA recruitment at the translocation site is sensed, when it is only necessary at a later stage in membrane protein assembly [64,69].

When translocation intermediates of secretory proteins are stuck in the inner membrane, they can be photo cross-linked to SecA and SecY [70]. Again, these interaction studies show that a similar or identical channel is used for both secretory and membrane proteins. The translocase seems to scan polypeptide chains for hydrophobic domains, and if amino acid stretches are sufficiently hydrophobic, translocation stops [71].

How do transmembrane segments move from the translocase into the lipid bilayer? In vitro crosslinking and in vivo depletion studies have identified a novel component that might be involved in this process: YidC [29,61,72]. At a nascent chain length of approximately 100 amino acids, the signal anchor sequence of two nascent model membrane proteins of different topology, FtsQ and Lep, were specifically photo cross-linked to YidC and to lipids while their hydrophilic regions were still close to SecA and SecY, suggesting that YidC is close to the translocase during membrane protein insertion. Similar observations were made for the multispanning membrane protein MtlA. The studies with MtlA indicated that YidC can simultaneously accommodate more than one transmembrane segment at the protein–lipid interface [65].

YidC could be co-purified with the Sec translocase and overexpression of translocase components positively affected YidC expression, suggesting a functional relationship [61]. YidC seems to interact with the SecDFYajC complex [73]. At least, the sequential interaction of the transmembrane segment of nascent FtsQ with SecY and YidC suggests that YidC acts after SecY has received the transmembrane segment in the membrane [61,63]. A similar order of interactions was detected in the mammalian ER with TRAM in the role of YidC. It remains to be established whether YidC and TRAM, which show no obvious similarity in primary sequence, are homologous in function [74]. Recently, besides a role for YidC in lipid partitioning of transmembrane segments, it has been suggested that YidC also plays a role in the reception of transmembrane segments at the membrane [75].

YidC is not only involved in the assembly of Sec-dependent membrane proteins. Depletion of YidC in a conditional strain not only affected the biogenesis of Sec-dependent membrane proteins but also of Sec-independent proteins like the small bacteriophage M13 procoat and Pf3 coat proteins [72,76,77]. It seems possible that YidC somehow facilitates lipid partitioning of the transmembrane segments of simple Sec-independent proteins without actually forming a translocation channel.

Interestingly, in the *E. coli* cell, most YidC molecules seem to be localized at the poles [64]. The key-role of YidC in membrane protein assembly in *E. coli* makes it tempting to speculate that in *E. coli* membrane proteins are synthesized at the poles of the cell and subsequently sorted to their final destination.

2.5. Assembly and folding of membrane proteins

Like in the ER, it is unclear whether transmembrane segments of polytopic membrane proteins that insert into the *E. coli* translocase move one by one into the lipid bilayer or en bloc after assembly and partial folding in the translocase [52]. Whatever the mechanism, the lipid bilayer is the final destination of a membrane protein. Evidence is accumulating that lipids have a more specific role than merely providing a solvent for membrane proteins [78,79]. Phosphatidylethanolamine appears to assist the folding of the membrane protein lactose permease. In this respect, lipids can be considered to function as molecular chaperones for membrane proteins.

Membrane proteins also contain cytoplasmic and periplasmic loops that might have to be correctly folded during or after synthesis. The role of chaperones in the folding of these loops has hardly been studied (see Ref. [80] for an example).

Besides the hydrophobic transmembrane segments, membrane proteins contain more information that determines their final structure and topology in the membrane. The distribution of positively charged amino acid residues is a major determinant of the transmembrane topology of membrane proteins [81]. The 'positive inside rule' states that regions of membrane proteins facing the cytoplasm are generally enriched in arginyl and lysyl residues, whereas translocated regions are largely devoid of these residues. It is still not clear how positively charged amino acid residues determine the topology of membrane proteins [82].

In systems other than *E. coli*, it has been shown that the binding of cofactors is sometimes needed for a membrane protein to reach its fully folded state. The binding of retinal to bacteriorhodopsin and of chlorophylls to the light-harvesting complex II are probably the best examples [83,84]. When membrane proteins are part of a complex, complex formation may also be needed for membrane proteins to reach their fully folded state.

2.6. Alternative membrane protein assembly pathways

Our knowledge of the biogenesis of membrane proteins in *E. coli* is based on monitoring the analysis of only a very limited number of (model) membrane proteins. It is very well possible that this has biased our ideas on membrane protein biogenesis. Evidence has emerged that the SRP/Sec translocase pathway is not the only membrane protein targeting/insertion pathway in *E. coli*. The bacteriophage M13 procoat and Pf3 coat proteins are the best known examples of membrane proteins that require neither the SRP targeting pathway nor any of the Sec components for their biogenesis [57,72,85].

In vivo depletion studies have provided evidence for a third membrane targeting/insertion pathway in *E. coli*: the SRP/YidC pathway [32]. It seems that in *E. coli*, the SRP targeting pathway can deliver membrane proteins directly to YidC, without involvement of any of the Sec components. In chloroplasts, which have a prokaryotic ancestor, a similar pathway is operational [86]. The chloroplast SRP can deliver membrane proteins without involvement of any of the CpSec components, to the thylakoidal membrane protein Albino3, which is the chloroplast homolog of YidC [87].

In *E. coli*, besides the Sec protein secretion pathway, another protein secretion pathway is operational, the T(win)A(rginine)T(ranslocation) pathway [88]. Pre-proteins transported by the TAT pathway usually bind redox cofactors and fold or even oligomerize before translocation across the membrane, whereas the Sec machinery can only accommodate noncompletely folded polypeptide chains. TAT substrates are characterized by an N-terminal signal peptide that contains a consensus "twin arginine" (RR) motif. So far, no clear example of a bacterial membrane protein targeted/inserted through the TAT pathway has been found. However, in chloroplasts, where a similar TAT pathway is operational, insertion of at least two thylakoidal membrane proteins that do not contain a twin arginine motif is mediated by the TAT pathway [89,90]. Thus, it cannot be ruled out that in *E. coli*, the TAT pathway assists the biogenesis of certain membrane proteins. In addition, it should be mentioned that, in principle, it is still possible that there are membrane proteins that can insert spontaneously (i.e., without the aid of protein factors) into the inner membrane (see e.g., Ref. [91]).

2.7. Concluding remarks

It is very likely that in *E. coli*, besides the SRP targeting pathway, there are alternative targeting mechanisms for membrane proteins. It is not known if one and the same Sec translocase is involved in the biogenesis of all Sec-dependent membrane proteins, or that there are different 'specialized' Sec translocases. It is also not known if Sec translocases are 'ready to use', or that Sec translocases are assembled during the membrane protein insertion process, depending on the needs of the membrane protein that is to be inserted into the inner membrane. It has been shown that secretory and membrane proteins engage different domains/components of the Sec translocase [59,92]. This probably explains why the biogenesis of membrane proteins, except the ones with large periplasmic domains, is not affected in *E. coli* mutant strains that are selected for protein secretion defects.

It appears that in *E. coli*, at least three different membrane protein targeting and insertion pathways exist: the SRP/Sec translocase pathway, the YidC pathway, and the SRP/YidC pathway [32]. The reason for and the relevance of these different membrane protein targeting and insertion pathways remain obscure.

So far, studies on the biogenesis of membrane proteins in *E. coli* have mainly focussed on identifying the components involved, and efforts have been made to reconstruct targeting and assembly pathways. It is very likely that not all components involved in membrane protein biogenesis have been identified yet, making it also possible that there are still unidentified membrane protein assembly pathways in *E. coli*.

Many important topics concerning the biogenesis of membrane proteins in *E. coli*, such as the role of the proton-motive force, lipids, the quality control of membrane protein biogenesis, the location of membrane protein biogenesis in the cell, cofactor binding, and the formation of membrane protein complexes have barely been touched yet. In a 'normal' *E. coli* cell, there are around 20.000–30.000 ribosomes, 40 SRP, 10.000 FtsY, 500 SecY/E/G, 2000 SecA, 30–40 SecD/F, and 2500 YidC molecules. At present, with our current knowledge of the assembly of membrane proteins in *E. coli*, we can only speculate as to

the reasons behind the differences in abundance of these components.

3. Membrane protein overexpression in *E. coli*

The overexpression of membrane proteins is an important bottleneck in studies of membrane protein function and structure (see, e.g., the Structural genomics supplement of *Nature Structural Biology*, November 2000). *E. coli* is one of the most widely used vehicles to overexpress both prokaryotic and eukaryotic (membrane) proteins since *E. coli* is very well accessible and easy to handle. Here we will only deal with the overexpression of membrane proteins. For more general information on the overexpression of proteins in *E. coli*, we refer to other review articles (see, e.g., Refs. [93,94]).

Is there a link between the overexpression and assembly of membrane proteins? Yes, since the overexpression of membrane proteins in a membrane is strongly favoured to the overexpression of membrane proteins in inclusion bodies. The reason being that it is relatively easy to isolate membrane proteins from a membrane, and usually very complicated to isolate them from inclusion bodies. There are only very few examples of membrane proteins that have successfully been isolated from inclusion bodies (see, e.g., Refs. [95,96]). It should be kept in mind that the overexpression of a membrane protein in a membrane does not necessarily mean that the membrane protein is also functional. The lipid composition of the membrane, cofactor binding, and other subunits when the membrane protein is part of a complex are among the factors that can determine if a membrane protein is functional or not.

At present, it is impossible to predict whether a membrane protein can be overexpressed. If the membrane protein can be overexpressed, it is not possible to predict if it will be overexpressed in inclusion bodies or in the membrane. This means that membrane protein overexpression is basically still a matter of 'trial and error'. To facilitate monitoring membrane protein overexpression in *E. coli* under many different conditions (e.g., under different culture conditions and in different strains), we have developed a green fluorescent protein (GFP) based membrane protein screen [97,98]. In short, GFP is fused to the C-terminus of the membrane protein in question. If the overexpressed GFP fusion ends up as inclusion bodies, GFP does not fold and is therefore not fluorescent. However, if the GFP fusion is expressed in the cytoplasmic membrane, GFP folds properly and is fluorescent. This screen speeds up the identification of membrane proteins that can be overexpressed well in *E. coli*, but does not help to improve the overexpression of membrane proteins that cannot or only poorly be (over)expressed. Hopefully, by studying the expression of a large number of membrane proteins, it may be possible to find some correlation between membrane proteins that 'express well' to those that 'express poorly'. It should be mentioned that the expression of membrane proteins does not seem to be affected by the GFP moiety [97], and that membrane proteins can easily be recovered from membrane protein GFP fusions [97]. Furthermore, it has been shown that the GFP moiety does not necessarily interfere with the functioning of a membrane protein (see, e.g., Refs. [64,99]).

Recently, *E. coli* mutant strains with improved (membrane) protein overexpression characteristics were isolated [100]. The way the strains were isolated indicates that they have accumulated multiple mutations, which have not yet been characterized. For the overexpression of membrane proteins, one very interesting observation has been made; upon the overexpression of a membrane protein, the intracellular membranes of one of these mutant strains can proliferate [101]. It is clear that membranes do not have an unlimited capacity to harbour membrane proteins and the proliferation of membranes could therefore explain the improved membrane protein overexpression yields.

The physiological consequences of the overexpression of membrane proteins in *E. coli* have hardly been studied, though they may give important clues as to why it is so difficult to overexpress membrane proteins. It has been shown that upon overexpression of membrane proteins, free SRP is titrated out [24]. Furthermore, in a recent study, G-protein-coupled receptors were successfully overexpressed in high quantities in a membrane system with an enormous membrane protein assembly capacity [102]. Together, these studies raise the question if the co-overexpression of factors involved in the assembly of membrane proteins would improve membrane protein overexpression yields. However, is our current knowledge of the assembly of membrane proteins sufficient to pursue co-overexpression strategies? Is it technically possible to co-overexpress the many factors that are involved in the assembly of membrane proteins in a concerted way? How does the cell react to the co-overexpression of factors involved in the assembly of membrane proteins? It is also very well conceivable that there are heterologous membrane proteins that are not the perfect substrates for the *E. coli* membrane protein assembly machinery. Tailoring of components for heterologous membrane protein overexpression is an intriguing but maybe not very realistic option.

Degradation of membrane proteins may also be an important factor that interferes with the overexpression of membrane proteins. Surprisingly, only very little is known about the degradation of membrane proteins in *E. coli*. However, there is one beautiful example of how a protease can interfere with the overexpression of a membrane protein in *E. coli*. When the Sec translocase component SecY is overexpressed, it is rapidly degraded by the FtsH protease [103]. However, SecY can be stably overexpressed when it is co-overexpressed with the Sec translocase component SecE, with which it forms a complex. A SecY derivative with a periplasmic PhoA moiety can be stably overexpressed; FtsH cannot pull this fusion out of the membrane

[104]. Thus, it is possible to stabilise overexpressed membrane proteins.

Taken together, membrane protein research would probably benefit a lot if the overexpression of membrane proteins would be considered as a science rather than an obligatory first step in studies of membrane proteins.

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